

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01N 47/24		A1	(11) International Publication Number: WO 99/09830
			(43) International Publication Date: 4 March 1999 (04.03.99)
(21) International Application Number: PCT/EP98/05701		(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HR, HU, ID, IL, IS, JP, KP, KR, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 18 August 1998 (18.08.98)			
(30) Priority Data: 60/056,638 22 August 1997 (22.08.97) US 60/079,628 27 March 1998 (27.03.98) US 98/04067 27 March 1998 (27.03.98) FR 60/084,477 6 May 1998 (06.05.98) US			
(71) Applicant: RHONE-POULENC AGRO [FR/FR]; 14-20, rue Pierre Baizet, F-69009 Lyon (FR).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: HOLMES, Keith, A.; 103 Exeter Court, Cary, NC 27511 (US). STAHL, Daniel, D.; 5229 Caber Road, Raleigh, NC 27612 (US).			
(74) Agent: TETAZ, Franck; Rhône-Poulenc Agro, Boîte postale 19163, F-69263 Lyon Cedex 09 (FR).			
(54) Title: METHOD OF INCREASING FOREIGN PROTEIN EXPRESSION			
(57) Abstract <p>A method of increasing protein expression encoded by a foreign gene in plants which comprises treating a locus comprising the plant or the seed from which it grows with aldicarb. A method of controlling pests at a locus, which locus comprises a plant containing a gene which encodes for <i>Bt</i> endotoxin, the method comprising the application of aldicarb at the plant or at the seed from which it grows. A method of controlling weeds at a locus, which locus comprises a plant containing a foreign gene which encodes a protein imparting tolerance to an herbicide, wherein the said herbicide is applied in said locus to said plant, the method comprising the application of aldicarb to the plant or to the seed from which it grows. A method of increasing the time during which an herbicide may be applied to an herbicide tolerant plant, which method comprises applying aldicarb to the said plant or to a seed from which it grows.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Method of Increasing Foreign Protein Expression

The present invention relates to a method of increasing foreign protein expression in plants, a method of controlling insects at a locus, a method of increasing the time during which an herbicide may be applied to a herbicide tolerant plant, a
5 plant in which the expression of a foreign protein has been increased by the application of aldicarb and a method of growing crops.

It is known in the field of plant biotechnology that heterologous genes encoding foreign proteins which impart herbicide tolerance, disease resistance or
10 toxicity to insects (insect resistance), or improve the quality of the crops, can be incorporated into the genome of certain plants. These genetically transformed plants can express these proteins for the added protection of the plants, or higher quality of the crops. Genes coding for protein imparting herbicide tolerance are known in the art, including genes imparting tolerance to oxynil herbicides (US 4,810,648 and US
15 5,559,024), genes imparting tolerance to glyphosate and EPSPS inhibitor herbicides (US 4,535,060, US 4,769,061, US 5,094,945, US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,312,910, US 5,310,667, US 5,633,435, US 5,627,061, US 5,554,798, US 5,633,448, WO 97/04103), genes imparting tolerance to glufosinate (EP 242 236), as well as genes imparting tolerance to HPPD inhibitors (WO 96/38567
20 and WO 98/02562). Genes coding for proteins imparting disease resistance are known in the art, including lytic peptides, oxalate oxydase genes for tolerance to sclerotinia or chitinases. Genes imparting insect resistance are also known in the art, including genes which encode for *Bacillus thuringiensis* (Bt) endotoxins (Inter alia, 5,460,963, 5,683,691, 5,545,565, 5,530,197, 5,317,096) etc., or for insecticidal toxins isolated
25 from *Photorhabdus* (WO 97/17432 or WO 98/08932). In general, these heterologous genes have been placed in a variety of plants including cotton.

It is also known that the levels of protein encoded by the heterologous genes in the transgenic plants can vary according to *inter alia* environmental conditions. For example, the grower of the said plants can be led to believe that the said plants are
30 capable of tolerance to herbicide without being substantially damaged by the

herbicide, or capable of resistance to diseases or withstanding attack by insect pests without further assistance from other pesticides. However, it is a severe problem for the grower if the said plants do not express the said protein at the correct time, and in an effective amount, particularly a pesticidally effective amount, more particularly an insecticidally effective amount in case of insect resistance. For example, in the case of *Bt* endotoxin, if there is not enough in the said plants, then the plants are susceptible to damage from pests, particularly lepidopteran pests, including bollworms. Cotton is such a plant that may be severely affected by bollworm attack. For another example, in the case of cotton seeds or plants comprising a gene encoding a Type II EPSPS, like Roundup Ready® Cotton, the window for application of EPSPS inhibitor herbicides is very strict and narrow (before the 4 leaves stage), and bolls may be damaged when the level of EPSPS expression is not sufficient to impart a proper level of tolerance to glyphosate, leading to an unacceptable loss of yield for the farmer.

Although it is known that aldicarb is a systemically acting insecticide, acaricide and nematocide and generally provides plants with a plant growth promoting effect, aldicarb is not generally effective against lepidopteran species when applied at the seed. Aldicarb is also known as 2-methyl-2-(methylthio)propionaldehyde O-(methylcarbamoyl)oxime.

An object of the present invention is to enhance the expression of heterologous proteins encoded by genes in plants.

Another object of the present invention is to enhance the expression of *Bacillus thuringiensis* endotoxin in plants.

Another object of the present invention is to enhance the expression of proteins imparting herbicide tolerance in plants.

Another object of the present invention is to provide an improved method for the control of pests at a locus.

Another object of the present invention is to provide an improved method of application of an herbicide to a plant tolerant to the said herbicide.

Another object of the invention is to provide an improved plant protected from

predacious insects.

Another object of the invention is to provide a plant with improved protection from herbivorous insects.

Another object of the invention is to provide an improved plant comprising a foreign gene which encodes for a foreign protein.

Another object of the invention is to provide an improved seed comprising a foreign gene which encodes for a foreign protein.

These objects are met in whole or in part by the present invention.

The present invention relates to a method of increasing foreign protein expression encoded by a foreign gene in plants comprising treating the plant or a seed from which it grows with aldicarb.

By the term "heterologous gene" or "foreign gene" is meant a gene that has been transformed or introduced into the plant, particularly integrated into the transformed plant genome, for example, transformed into the plant or an ancestor thereof, more particularly a gene that is not naturally present in the transformed plant, or even more particularly a gene which contains a sequence coding for a heterologous or foreign protein, including regulatory elements appropriate for controlling the expression of the said coding sequence in the plant cells, for example, promoters, terminators, pre-pro peptides, or transit peptides, the latter driving the expression of the said heterologous protein in a specific targeted region of the plant cell.

Promoters controlling the expression of a gene in plant cells are well known in the field of plant biotechnology, including any promoter sequence of a gene naturally expressed in plants or plant cells, from plant, viral or bacterial origin. Suitable such promoters are disclosed in Weising & al (1988, Annual Rev. Genet., 22 :241, the subject matter of which is incorporated herein by référence). The following is a partial representative list of promoters suitable for use herein : regulatory sequences from the T-DNA of *A. tumefaciens*, including mannopine synthase, nopaline synthase and octopine synthase ; regulatory sequences from plant origin, including alcohol deshydrogenase promoter from corn, light inducible promoters such as ribulose-biscarboxylase/oxygenase small subunit promoters (SSU

RuBisCO) from genes of a variety of species and the major chlorophyll a/b binding gene promoters, histone promoters (EP 507 698), actin promoters, maize ubiquitin 1 promoters (Christenses & al., 1996, *Transgenic Res.*, 5 :213) ; regulatory sequences from viral origins, such as 19S or 35S promoters of the cauliflower mosaic virus, (US 5,352,605 ; US 5,530,196) ; developmentally regulated promoters such as waxy, zein, or bronze promoters from maize ; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant, like the promoter of napin (EP 255 378) or the alpha-tubulin promoter (US 5,635,618).

By the term "heterologous protein" or "foreign protein" is meant a protein of interest produced by the expression of the foreign gene in the plants. It may be a protein naturally present in the transformed plant specie but expressed at levels or in specific plant tissues or targeted regions of the plant cells, not naturally occurring in the said transformed plant specie, or a protein not naturally present in the transformed plant specie.

The invention provides a method of increasing foreign gene expression in transgenic plants which method comprises treating the plant or a seed from which it grows with aldicarb.

The transgenic plants according to the invention include cotton, corn, potato, soybean, sunflower, rape seed oil, sugar cane, sugar beet, rice, alfalfa, or banana plants, preferably cotton, corn or potato plants, most preferably a cotton plant.

Preferably, the foreign genes according to the present invention are all the foreign or heterologous genes coding for proteins of interest to impart herbicide tolerance , disease resistance or toxicity to insects (insect resistance), or to improve quality of the crops, known in the art and/or disclosed here above.

As a preferred embodiment of the present invention, the heterologous or foreign gene encodes a protein to impart herbicide tolerance, more preferably tolerance to an oxynil herbicide (disclosed in US 4,810,648 and US 5,559,024, incorporated herein by reference), to EPSPS inhibitor herbicides, including glyphosate and its various salts (disclosed in US 4,535,060, US 4,769,061, US 5,094,945, US

4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,312,910, US 5,310,667, US 5,633,435, US 5,627,061, US 5,554,798, US 5,633,448, WO 97/04103, incorporated herein by reference), to glufosinate (EP 242 236, incorporated herein by reference), or to HPPD inhibitors (WO 96/38567 and WO 98/02562, incorporated
5 herein by reference). More preferably, the heterologous or foreign gene encodes a protein to impart tolerance to EPSPS inhibitor herbicides.

As another preferred embodiment of the present invention, the heterologous or foreign gene encodes a protein to impart insect resistance, more preferably genes which encode for *Bacillus thuringiensis* (*Bt*) endotoxins (Inter alia, US 5,460,963, US
10 5,683,691, US 5,545,565, US 5,530,197, US 5,317,096, incorporated herein by reference). The genes that are preferably embraced by the instant invention are *cryI*, *cryII*, *cryIII*, and *cryIV* genes. More preferably, the genes include: *cryIA(a)*; *cryIA(b)*; *cryIA(c)*; and *cryIIIA(a)*. Most preferably the gene is *cryIA(a)*, *cryIA(b)* or *cryIA(c)*. The type of plant which expresses a *Bt* endotoxin is known as a "Bt" plant, for
15 example "Bt-cotton", "Bt-corn" or "Bt-potato."

The promoters controlling the expression of the above genes in plant cells include the following : regulatory sequences from the T-DNA of *A. tumefaciens*, including mannopine synthase, nopaline synthase and octopine synthase ; regulatory sequences from plant origin, including alcohol deshydrogenase promoter from corn,
20 light inducible promoters such as ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species and the major chlorophyl a/b binding gene promoters, histone promoters (EP 507 698), actin promoters, maize ubiquitin 1 promoters (Christensen & al., 1996, *Transgenic Res.*, 5 :213) ; regulatory sequences from viral origins, such as 19S or 35S promoters of the
25 cauliflower mosaic virus, (US 5,352,605 ; US 5,530,196) ; developmentally regulated promoters such as waxy, zein, or bronze promoters from maize ; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant, like the napin promoter (EP 255 378) or the alpha-tubulin
30 promoter (US 5,635,618). As a preferred embodiment, the promoter is selected

among the group consisting in the ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species, the histone promoters, the actin promoters, the maize ubiquitin 1 promoters and the 35S promoters of the cauliflower mosaic virus (CaMV 35S), most preferably the CaMV 35 S promoter.

5 The transgenic plant may be a *Bt*-plant in which the foreign gene expresses *Bt*-endotoxin and/or an herbicide tolerant plant in which the foreign gene expresses a foreign protein imparting tolerance to the said herbicide.

Preferably the transgenic plants are cotton plants, most preferably NuCotn® 32B or NuCotn® 33B or those comprising a foreign gene which encode the *cryIA(a)*
10 or *cryIA(c)* genes, BXN® Cotton comprising a foreign gene which encodes a nitrilase for oxynil tolerance, or Roundup Ready® Cotton comprising a foreign gene which encodes for an EPSPS for glyphosate tolerance.

The foreign gene expression can be elevated in any part of the plant, depending upon the result to be achieved. For herbicide tolerance, the foreign gene
15 should be expressed at high levels in all the parts of the plants, more particularly in the green tissues of the transformed plants, e.g., the leaves and reproductive structures. For insecticidal activity, particularly *Bt*, or herbicide tolerance, the foreign gene may be expressed preferably in the fruiting parts of the cotton plant, most preferably the squares or bolls and may depend on the exact variety of the plant.

20 Aldicarb may be applied directly to the plant and/or to the seed and/or to a locus where the plant is growing or is to be grown. By the term "to the seed" is meant on or near the seed.

In one embodiment, aldicarb may be applied to the seed in a seed treatment step before or just after planting. That is, the aldicarb and the seed may be placed in
25 the ground simultaneously or sequentially.

Aldicarb may be applied to the locus before or after planting. The plants may be treated with aldicarb by known methods of application such as side-dressing or in-furrow application of granules or liquid; or by drilling.

In one other embodiment, aldicarb may be applied to the locus after planting
30 but before emergence of the plant.

In a preferred aspect of the invention, the seeds are treated after planting with aldicarb at a rate of from 0.01 to 6 kg/ha (kilograms of active ingredient per hectare) preferably from 0.05 to 4 kg/ha more preferably from 0.1 to 1 kg/ha. Generally the amount of aldicarb per 100 meters of crop row is of from 10 to 500 g/100m (grams per 100 meters), preferably from 20 to 100 g/100m.

In another preferred embodiment of the invention, the seeds after treatment with aldicarb before planting may comprise from 0.001 % w/w to 20% w/w of aldicarb to seed, preferably from 0.05% to 10%, more preferably from 0.1% to 5%.

The present invention also relates to a method of controlling pests at a locus, which locus comprises a plant containing a gene which encodes for *Bt* endotoxin, the method comprising the application of aldicarb to the plant or to the seed from which it grows.

It has been unexpectedly found that the application of aldicarb to the plant or to the seed from which it grows improves the protection of the plant from attack by pests, preferably insect pests, more preferably of the Orders Lepidoptera, Coleoptera or Diptera. More preferably, the pests are of the family Noctuidae, even more preferably *Heliothis* spp. or *Helicoverpa* spp. Another pest species controlled by the method of the instant invention is *Pectinophora* spp particularly *Pectinophora gossypiella*. Specific pests controlled by the instant invention include at least one of the following species: *Helicoverpa armigera* (also known as *Heliothis armigera*); *Helicoverpa virescens* (also known as *Heliothis virescens* or Tobacco budworm); *Heliothis punctigera*; *Heliothis zea* (cotton bollworm); *Heliothis assulta*; and *Heliothis peltigera*.

Other Lepidoptera that may be controlled by the method of the present invention include *Ostrinia nubilalis*, *Spodoptera littoralis*, *Spodoptera exigua*, *Spodoptera frugiperda*, *Manduca sexta*, *Pieris rapae*, *Trichoplusia ni*, and *Alabama argillacea*.

Coleoptera that may be controlled by the method of the present invention include the family Crysomelidae, particularly *Leptinotarsa decemlineata* and *Diabrotica undecimpunctata howardi*.

Diptera that may be controlled by the method of the present invention include insects of the family Phorbia, particularly *Phorbia brassicae* and *Phorbia platura*.

The transgenic Bt-plants according to the invention include cotton, corn, potato, sugar cane or rice plants, preferably cotton, corn or potato plants, most
5 preferably a cotton plant.

In a highly preferred aspect of the instant invention, it has been unexpectedly found that in Bt-cotton plants treated with aldicarb, the control of certain species of lepidoptera, most preferably *Heliothis armigera*, is higher than in those Bt-cotton plants not treated with aldicarb. The Bt-cotton in the instant invention is most
10 commonly known commercially as NuCotn 32B or NuCotn 33B or Ingard.

The present invention also relates to a method of controlling weeds at a locus, which locus comprises a plant containing a foreign gene which encodes a protein imparting tolerance to an herbicide, wherein the said herbicide is applied in said locus to said plant, the method comprising the application of aldicarb to the plant or to the
15 seed from which it grows.

The present invention also relates to a method of increasing the time during which an herbicide may be applied to an herbicide tolerant plant, which method comprises applying aldicarb to the said plant or to a seed from which it grows.

As a preferred embodiment, the herbicide is an EPSPS inhibitor herbicide, including glyphosate and its various salts, like the isopropylammonium salt or the sulfosate salt (i.e. Roundup® or Touch Down ®), and the foreign gene encodes for a protein imparting tolerance to the said EPSPS inhibitor herbicide.
20

It has been unexpectedly found that the application of aldicarb, not only increases the tolerance to the herbicide of the transgenic plant, but also increase the time during which the glyphosate may be applied in said locus to said plant (glyphosate being understood as the representative of the whole family of EPSPS inhibitor herbicides).
25

The transgenic herbicide tolerant according to the invention include cotton, corn, soybean, sunflower, rape seed oil, sugar cane, sugar beet or alfalfa plants, preferably cotton, corn or soybean plants, most preferably a cotton plant.
30

The number of applications of EPSPS inhibitor herbicides, including glyphosate or a salt thereof, per growing season may also be increased, in general from 1 to 7 sprays per season, preferably 2 to 6 sprays per season, more preferably 2 to 5 sprays per season.

5 In the case of cotton, glyphosate or a salt thereof may be applied on the plant from the emergence of the plant to harvest of the cotton, preferably from emergence to boll opening, more preferably from emergence to the 12 leaf stage, even more preferably from emergence to the 8 leaf stage, even more preferably from emergence to the 6 leaf stage.

10 The present invention also relates to a new composition which comprises aldicarb and a seed containing a foreign gene which encodes for a foreign protein. The foreign gene and the seeds of the plant may be one of those disclosed above. The composition may comprise from 0.001 % w/w to 20% w/w of aldicarb to seed, preferably from 0.05% to 10%, more preferably from 0.1% to 5%. The aldicarb may
15 coat the seed in a conventional manner as known to the skilled addressee in the art of seed coatings. In addition, the aldicarb may be injected into the seed before planting.

 The present invention also relates to a plant which comprises a foreign gene which encodes for a foreign protein as disclosed here above which has been treated by aldicarb, the said plant having an improved amount of foreign protein.

20 In the case of insect tolerance and *Bt* endotoxin, the plant preferably has more endotoxin in the fruiting parts of the plant than in the leaves; in the case of cotton, in the bolls and squares than in the leaves.

 Aldicarb, its uses and formulations are described inter alia in The Pesticide Manual, 10th edition, Crop Protection Publications, ed. C. Tomlin, Bath, United
25 Kingdom and Crop Protection Reference 11th edition, CPR Press, New York, NY, USA, 1995. Plants and descriptions of plants that express *Bacillus thuringiensis* delta endotoxin are available from the Monsanto® and Novartis® agrochemical companies. Plants and description of plants that express genes imparting tolerance to an EPSPS inhibitor herbicide are available from the Monsanto® agrochemical company. Salts of
30 glyphosate are available from the Monsanto® and Zeneca® agrochemical companies.

The following examples illustrate the invention.

EXAMPLE 1:

Seeds of NuCotn® 32B are planted in a field. Aldicarb, in the form of the commercial product Temik® 15G (a 15% weight/weight granular formulation) is applied in the furrow that comprises the seed at a rate of about 0.5 kg/ha of active ingredient or 3.3 kg/ha of commercial product. At various points in the growing season, the plant tissues are tested for the levels of the delta-endotoxin. The tissues from the plants treated by aldicarb display a higher level of the endotoxin than the tissues from plants not treated by aldicarb.

EXAMPLE 2:

Seeds of NuCotn® 32B are planted in a field. Aldicarb, in the form of the commercial product Temik® 15G (a 15% weight/weight granular formulation) is applied as a side-dress at 7.8 kg/ha of commercial product. After a full growing season, there are a larger number of bolls on the plants that have been treated with aldicarb in comparison to those not treated with aldicarb. The amount of damage due to bollworm attack on the cotton is less in those treated with aldicarb in comparison to those not treated with aldicarb.

EXAMPLE 3:

Cotton leaves and squares were collected from treatments of Bt Cotton wherein the cotton was treated with Temik®15G under the following conditions;

- A) 0.45 lbs active ingredient per acre at plant (0.5 kg/ha)
- B) 0.75lbs active ingredient per acre at plant; (0.5 kg/ha) and
- C) 0.75 lbs active ingredient per acre (0.84 kg/ha) at plant and followed 3 weeks later by a side-dress treatment of 1.0 lbs per acre (1.12 kg/ha) of active ingredient.

One planting was not treated with aldicarb.

After 90 days leaves and squares were collected, washed with soap and water,

allowed to dry, washed with acetone, allowed to dry and finally washed with chloroform. The tissues were then freeze dried, ground in a mortar and pestle and stored at room temperature.

5 The cotton tissues were incorporated into a *Heliothis* diet and fed to *Heliothis virescens*. The larvae in the cups were weighed at seven days after infestation and the number of pupae were counted 14 and 21 days after infestation. The percent pupation was calculated at the different concentrations and the EC_{50P} (the amount of tissue per liter of diet that reduced pupation by 50%) was calculated for each insecticide
10 treatment to express the amount of toxin activity in the treatment.

At the reading two weeks after infestation, the diet containing leaf tissue of cotton plants treated with aldicarb at plant required higher amounts of tissue to reduce pupation by 50% compared to the untreated check, but the diet containing square
15 tissue required lesser amounts of tissue to reduce pupation by 50%.

At the reading three weeks after infestation, the diet containing leaf tissue of cotton plants treated with aldicarb at plant required higher amounts of tissue to reduce pupation by 50% compared to the untreated check, but the diet containing square
20 tissue required lesser amounts of tissue to reduce pupation by 50%.

This indicates that the Bt endotoxin is at least preferably accumulating more in the squares of the Bt cotton plant when treated with aldicarb.

25 **EXAMPLE 4:**

V2 INGARD Cotton was treated with a Temik®15G at plant at a rate of 5 kg per hectare of commercial product. After 3 weeks leaves from the 3rd node were picked and fed to *Heliothis armigera* 1st instar larvae. At this time the cotton leaves from the third node in the treated plants were less effective than the leaves from the
30 untreated plants.

Two weeks later leaves from the 3rd node and 7th node were taken from treated and untreated plants. Squares were also sampled. When fed to *Heliothis armigera* larvae, there was a higher mortality in the Temik-treated plant tissue than for the untreated plant tissue. Furthermore, the surviving larvae were significantly smaller when fed the treated leaves than the untreated leaves.

EXAMPLE 5

A plot of Roundup Ready® Cotton (RRC) was grown up. One part of the plot was treated with Temik® at a rate of 5.6 kg/ha of commercial product at the time of planting of the seed. Another part was treated with Disyston® (disulfoton) at 22.24 kg of commercial product/ha the time of planting of the seed. Another part of the plot was grown from seed treated with Gaucho® (imidacloprid) at a rate of 0.357 kg of commercial product per 100 kg of seed. One part of the plot was left untreated by an insecticide which is called hereafter the untreated check (UTC).

The isopropylamine salt of glyphosate was applied to all the cotton plants at the four-leaf stage in the form of the commercial product Roundup® at a rate of 32 oz of commercial product per acre (= 0.17 kg/ha of active ingredient). The following results were observed 45 days after the Roundup® treatment:

Treatment	Squares per Plant	% change from UTC
RRC (UTC)	7.45	----
RRC + aldicarb	9.05	+21
RRC + disulfoton	7.65	+2
RRC + imidacloprid	8.70	+18

EXAMPLE 6

Roundup Ready® Cotton was grown up as in Example 5 and treated with Roundup at the six-leaf stage.

The following results were observed 40 days after the Roundup® treatment:

Treatment	Squares per Plant	% change from UTC
RRC (UTC)	8.85	-----
RRC + aldicarb	10.95	+23%
RRC + disulfoton	5.65	-36%
RRC + imidacloprid	7.60	-14%

The observations of Examples 5 and 6 were made on the same day.

The results of Examples 5 and 6 show that the treatment with aldicarb not only increases the yield of cotton compared with UTC or cotton treated with other herbicides, but also that the time during which glyphosate may be applied without damage to the plant is increased after the four leaf stage, with the same yield improvement, when treatment with other herbicides leads to a decrease of yields.

CLAIMS

1. A method of increasing foreign protein expression encoded by a foreign gene in plants comprising treating the plant or a seed from which it grows
5 with aldicarb.

2. The method according to claim 1, wherein the plant is selected among the group consisting of cotton, corn, potato, soybean, sunflower, rape seed oil, sugar cane, sugar beet, rice, alfalfa, and banana plants, preferably cotton, corn or potato plants, most preferably a cotton plant.

10 3. The method according to one of claims 1 or 2, wherein the foreign genes are foreign or heterologous genes coding for proteins of interest to impart herbicide tolerance, disease resistance, toxicity to insects (insect resistance), or to improve quality of the crops.

4. The method according to claim 3, wherein the heterologous or foreign
15 gene encodes a protein to impart herbicide tolerance.

5. The method according to claim 4, wherein heterologous or foreign gene encodes a protein to impart tolerance to an herbicide selected among the group consistin of oxynil herbicide, EPSPS inhibitor herbicides, including glyphosate and its various salts, glufosinate and HPPD inhibitor herbicides

20 6. The method according to claim 5, wherein the heterologous or foreign gene encodes a protein to impart tolerance to EPSPS inhibitor herbicides.

7. The method according to claim 3, wherein the heterologous or foreign gene encodes a protein to impart insect resistance.

8. The method according to claim 7, wherein the heterologous or foreign
25 gene encodes for *Bacillus thuringiensis* (Bt)

9. The method according to claim 8, wherein the heterologous or foreign gene is selected among the group consisting of *cryI*, *cryII*, *cryIII*, and *cryIV* genes.

10. The method according to claim 9, wherein the heterologous or foreign gene is selected among the group consisting of: *cryIA(a)*; *cryIA(b)*; *cryIA(c)*; and
30 *cryIIIA(a)* genes.

11. The method according to one of claims 1 to 10; wherein the promoter controlling the expression of the heterologous or foreign gene is selected among the group consisting in the ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species, the histone promoters, the actin
5 promoters, the maize ubiquitin 1 promoters and the 35S promoters of the cauliflower mosaic virus (CaMV 35S).

12. The method according to claim 11, wherein the promoter is the CaMV 35 S promoter.

13. The method according to one of claims 1 to 12, wherein aldicarb is
10 applied directly to the plant and/or to the seed and/or to a locus where the plant is growing or is to be grown.

14. The method as claimed in claim 13, wherein aldicarb is applied to the seed in a seed treatment step before or just after planting, simultaneously or sequentially.

15. The method according to claim 13, wherein aldicarb is applied to the locus before or after planting.

16. The method as claimed in claim 15, wherein aldicarb is applied at the locus after planting but before emergence of the plant.

17. The method as claimed in one of claims 1 to 16, wherein the seeds are
20 treated after planting with aldicarb at a rate of from 0.01 to 6 kg/ha (kilograms of active ingredient per hectare) preferably from 0.05 to 4 kg/ha more preferably from 0.1 to 1 kg/ha.

18. The method as claimed in one of claims 1 to 14, wherein the seeds after treatment with aldicarb before planting comprise from 0.001 % w/w to 20% w/w
25 of aldicarb to seed, preferably from 0.05% to 10%, more preferably from 0.1% to 5%.

19. A method of controlling pests at a locus, which locus comprises a plant containing a gene which encodes for *Bt* endotoxin, the method comprising the application of aldicarb to the plant or to the seed from which it grows.

20. The method according to claim 19, wherein the pest controlled is from
30 the Orders Lepidoptera, Coleoptera or Diptera.

21. The method according to one of claims 19 to 20, wherein plant is selected among the group consisting of cotton, corn, potato, sugar cane and rice plants, preferably cotton, corn and potato plants, most preferably a cotton plant.

22. The method according to claim one of claims 19 to 21, wherein the
5 heterologous or foreign gene is selected among the group consisting of *cryI*, *cryII*, *cryIII*, and *cryIV* genes.

23. The method according to claim 22, wherein the heterologous or foreign gene is selected among the group consisting of: *cryIA(a)*; *cryIA(b)*; *cryIA(c)*; and *cryIIIA(a)* genes.

10 24. The method according to one of claims 19 to 23, wherein the promoter controlling the expression of the heterologous or foreign gene is selected among the group consisting in the ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species, the histone promoters, the actin promoters, the maize ubiquitin 1 promoters and the 35S promoters of the cauliflower
15 mosaic virus (CaMV 35S).

25. The method according to claim 24, wherein the promoter is the CaMV 35 S promoter.

26. The method according to one of claims 19 to 25, wherein aldicarb is applied directly to the plant and/or to the seed and/or to a locus where the plant is
20 growing or is to be grown.

27. The method as claimed in claim 26, wherein aldicarb is applied to the seed in a seed treatment step before or just after planting, simultaneously or sequentially.

28. The method according to claim 26, wherein aldicarb is applied to the
25 locus before or after planting.

29. The method as claimed in claim 28, wherein aldicarb is applied at the locus after planting but before emergence of the plant.

30. The method as claimed in one of claims 19 to 29, wherein the seeds are treated after planting with aldicarb at a rate of from 0.01 to 6 kg/ha (kilograms of
30 active ingredient per hectare) preferably from 0.05 to 4 kg/ha more preferably from

0.1 to 1 kg/ha.

31. The method as claimed in one of claims 19 to 28, wherein the seeds after treatment with aldicarb before planting comprise from 0.001 % w/w to 20% w/w of aldicarb to seed, preferably from 0.05% to 10%, more preferably from 0.1% to 5%.

5 32. A method of controlling weeds at a locus, which locus comprises a plant containing a foreign gene which encodes a protein imparting tolerance to an herbicide, wherein the said herbicide is applied in said locus to said plant, the method comprising the application of aldicarb to the plant or to the seed from which it grows.

10 33. A method of increasing the time during which an herbicide may be applied to an herbicide tolerant plant, which method comprises applying aldicarb to the said plant or to a seed from which it grows.

34. The method as claimed in one of claims 32 or 33, wherein the herbicide tolerant plant is selected among cotton, corn, soybean, sunflower, rape seed oil, sugar cane, sugar beet or alfalfa plants, preferably cotton, corn or soybean plants,
15 most preferably a cotton plant.

35. The method as claimed in one of claims 32 to 34, wherein the herbicide is an EPSPS inhibitor herbicide, including glyphosate and its various salts, like the isopropylammonium salt or the sulfosate salt, and the foreign gene encodes for a protein imparting tolerance to the said EPSPS inhibitor herbicide.

20 36. The method according to one of claims 32 to 35, wherein the promoter controlling the expression of the heterologous or foreign gene is selected among the group consisting in the ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species, the histone promoters, the actin promoters, the maize ubiquitin 1 promoters and the 35S promoters of the cauliflower mosaic virus (CaMV 35S).
25

37. The method according to claim 36, wherein the promoter is the CaMV 35 S promoter.

38. The method according to one of claims 32 to 37, wherein aldicarb is applied directly to the plant and/or to the seed and/or to a locus where the plant is
30 growing or is to be grown.

39. The method according to claim 38, wherein aldicarb is applied to the seed in a seed treatment step before or just after planting, simultaneously or sequentially.

40. The method according to claim 38, wherein aldicarb is applied to the
5 locus before or after planting.

41. The method as claimed in claim 41, wherein aldicarb is applied at the locus after planting but before emergence of the plant.

42. The method as claimed in one of claims 32 to 41, wherein the seeds are treated after planting with aldicarb at a rate of from 0.01 to 6 kg/ha (kilograms of
10 active ingredient per hectare) preferably from 0.05 to 4 kg/ha more preferably from 0.1 to 1 kg/ha.

43. The method as claimed in one of claims 32 to 39, wherein the seeds after treatment with aldicarb before planting comprise from 0.001 % w/w to 20% w/w of aldicarb to seed, preferably from 0.05% to 10%, more preferably from 0.1% to 5%.

15 44. A new composition which comprises aldicarb and a seed containing a foreign gene which encodes for a foreign protein.

45. The composition according to claim 44, wherein it comprises from 0.001 % w/w to 20% w/w of aldicarb to seed, preferably from 0.05% to 10%, more preferably from 0.1% to 5%.

20 46. A plant which comprises a foreign gene which encodes for a foreign protein as disclosed in one of the preceding claims, wherein the said plant has an improved amount of foreign protein.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/05701

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N47/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. TOMLIN (ED): "The Pesticide Manual" , BRITISH CROP PROTECTION COUNCIL , UK XP002091614 10th edition, 1994 see page 24 - page 26 -----	

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

29 January 1999

Date of mailing of the international search report

17/02/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Decorte, D

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/EP 98/05701

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N47/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. TOMLIN (ED): "The Pesticide Manual" , BRITISH CROP PROTECTION COUNCIL , UK XP002091614 10th edition, 1994 see page 24 - page 26 -----	

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

29 January 1999

Date of mailing of the international search report

17/02/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Decorte, D